Protein aggregation in *Escherichia coli*: role of proteases

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Abstract

Protein aggregation is involved in several human diseases, and presumed to be an important process in protein quality control. In bacteria, aggregation of proteins occurs during stress conditions, such as heat shock. We studied the protein aggregates of *Escherichia coli* during heat shock. Our results demonstrate that the concentration and diversity of proteins in the aggregates depend on the availability of proteases. Aggregates obtained from mutants in the Lon (La) protease contain three times more protein than wild-type aggregates and show the broadest protein diversity. The results support the assumption that protein aggregates are formed from partially unfolded proteins that were not refolded by chaperones or degraded by proteases. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Heat shock; Lon (La) protease; ClpP protease; HslVU protease

1. Introduction

The study of protein aggregation and the ways to control it is of high interest in recent years. One of the current aspects of interest arises from the involvement of protein aggregation in a variety of unrelated diseases, including Alzheimer’s disease, bovine spongiform encephalopathy, and type II diabetes [1–3]. Protein aggregation probably results from interaction between exposed hydrophobic surfaces of neighboring molecules to produce large multi-molecular ensembles. These hydrophobic surfaces, normally buried in the core of the proteins, are exposed following unfolding. It has been assumed that cellular misfolded proteins are subjected to either refolding by chaperones or degradation by proteases [4,5]. A misfolded protein, which escapes these quality control mechanisms, may interact with other unfolded proteins to form aggregates. Protein aggregates are considered ‘dead ends’, as they are probably non-functional and inaccessible to proteases and most chaperones. Yet, sometimes they may cause severe damage to the organism, as indicated by their involvement in diseases [6].

In *Escherichia coli* the cellular system that copes with de novo protein folding and quality control consists of the molecular chaperones (DnaK, DnaJ and GrpE; GroEL and GroES; HtpG; IbpA and IbpB) and proteases (ClpX, ClpP; HslU, HslV; Lon; FtsH) [4,7]. The molecular chaperones operate under all growth conditions and become particularly important under stress conditions such as heat shock, during which they are also induced [4,8–10]. In mutants lacking the heat shock sigma factor (σ2) there is an increase in the concentration of aggregates, which also occurs in mutants lacking DnaK or GroEL [4]. These results support the idea that the aggregates represent proteins that could not be refolded. Since such proteins are presumably degraded by cellular proteases, we assumed that there will be an increase in protein aggregation in mutants lacking activity of these proteases. Indeed, the results show that aggregates from *E. coli* strains lacking any one of the major cytosolic proteases contain more protein than aggregates from wild-type *E. coli*. Moreover, analysis of 2D gel electrophoresis of the proteins from the aggregates demonstrates a much larger variety of proteins in mutant strains. We assume that the proteins in the aggregates represent the natural substrates of each of the proteases. Therefore, comparison
of the aggregates proteome of wild-type and mutants strains can be used to identify protease-specific target proteins.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Table 1 lists the bacterial strains used. Bacteria were grown at 30°C in Davis and Mingioli minimal medium [11] supplemented with 0.2% glucose. Heat shock was achieved by transfer of exponentially growing cells (about $4 \times 10^8$ bacteria per ml) from 30°C to 42°C for 60 min.

2.2. Isolation of aggregated proteins

Isolation of aggregated proteins was based on the method described in [4]. Aliquots (100 ml) of bacterial cultures were rapidly cooled to 0°C in an ice-water bath. The cells were centrifuged and washed twice with TE-PMSF (10 mM Tris pH 7.5; 1 mM EDTA; 1.4 mM phenylmethylsulfonyl fluoride). The washed cells were resuspended in 0.5 ml of TE-PMSF and disrupted by Microson sonicator (microtip, level 6, 3 min 50% duty). The extracted proteins were then centrifuged at 20,000 $\times$ g for 30 min at 4°C, the pellet was resuspended in 0.5 ml of TE-PMSF and disrupted by Microson sonicator. The insoluble fraction (containing membranes and aggregated proteins) was isolated by subsequent centrifugation at 20,000 $\times$ g for 30 min at 4°C. The pellet fractions were resuspended in 500 µl TE-PMSF-2% NP40 by brief sonication and the aggregated proteins were precipitated by centrifugation (20,000 $\times$ g for 30 min at 4°C). This washing procedure was repeated to allow the complete removal of contaminating membrane proteins. NP40-insoluble pellets were resuspended in gel rehydration solution (8 M urea; 2 M thiourea; 5.2 µl ml$^{-1}$ Pharmalytes (pH 3-10); 10 mg ml$^{-1}$ CHAPS (Sigma Chemicals Co.) and 2 mg ml$^{-1}$ dithiothreitol) by brief sonication.

The protein contents of the soluble fractions and of the aggregated proteins were determined by the Bradford method [12].

2.3. Proteome analysis

Two-dimensional polyacrylamide gel electrophoresis and protein identification were done according to Büttner et al. [13].

3. Results and discussion

3.1. Effect of deletions in genes encoding proteases on the concentration of aggregates

To investigate the role of E. coli proteases in preventing protein aggregation during heat shock conditions, we quantified the aggregated proteins from wild-type E. coli and three mutant strains lacking one of the cytosolic proteases Lon, ClpP or HslVU.

Protein aggregates were purified from cell extracts by the newly described method of Tomoyasu et al. [4]. The protein content of the aggregates and the total protein of the samples were then determined [12] and the fraction of aggregated proteins was calculated (Fig. 1). Mutants in Lon and ClpP proteases contained higher concentrations of aggregates. The highest concentration was in aggregates of E. coli JT 4000, null mutant in the lon gene, suggesting a major role for the Lon protease in protein quality control. This finding is in agreement with the fact that Lon becomes essential at 42°C in DnaK-depleted cells [4], where there is a substantial reduction in protein refolding. Surprisingly, there was no increase in protein concentration in the aggregates of mutants lacking the HslVU protease. This result indicates that quantitatively most of the proteolysis of unfolded proteins is carried out by the Lon and ClpP proteases. These results are in agreement with

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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<tbody>
<tr>
<td>E. coli K12</td>
<td></td>
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<tr>
<td>MG 1655</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JT 4000</td>
<td>MC4100 $\Delta$lon510, lac</td>
<td>[18]</td>
</tr>
<tr>
<td>SG22175</td>
<td>MC4100 $clpP::kan$ (polar on $clpX$)</td>
<td>[19]</td>
</tr>
<tr>
<td>DM1674</td>
<td>MC4100 $hslV$ $hslU::6\text{Cm}$</td>
<td>[20]</td>
</tr>
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![Fig. 1. Relative levels of protein in aggregates. Cultures of wild-type E. coli K12 and mutants in the lon, clpP or hslVU genes were grown as described in Section 2.1. When the cell density reached about $4 \times 10^8$ bacteria per ml they were treated as described in Section 2.2. The concentration of the soluble and aggregated proteins was determined [12] and the fraction of aggregates from the total proteins was calculated. The fraction of aggregated proteins in the wild-type was taken as 1.](image-url)
the finding that Lon and ClpP are responsible for 80% of the ATP-dependent proteolysis [14].

3.2. Proteome analysis of aggregated proteins

To further investigate the role of each protease in aggregate formation, we performed 2D proteome analysis of aggregates from wild-type cells as well as from mutants lacking proteases. Equal amounts of aggregated proteins from the wild-type and from three mutant strains heat-shocked at 42°C for 60 min were separated using 2D gel electrophoresis. Comparison of the proteome of aggregated protein wild-type and *lon* mutant strain (Fig. 2) shows a considerably different protein pattern. In the aggregated proteins from wild-type MG1655 strain the major protein is elongation factor-Ts. This protein constitutes more than 50% of the aggregated protein content. In contrast, in the *lon* mutant strain the pattern of aggregated proteins on a 2D gel is very similar to that of the soluble cytosolic proteins (compare Fig. 2A and C), indicating that most of the cellular proteins become misfolded as a result of heat shock. We identified several of these proteins, as listed in Table 2. These include metabolic enzymes (BPG, DAHP synthase and LDH), proteins from the protein synthesis system (elongation factor-Ts and ribosome recycling factor) and others. We could also identify in the aggregates the two major chaperones — DnaK and GroEL. These results support the suggestion that following heat shock protein unfolding leading to aggregation is a general process, involving the majority, if not all, of the proteins.

The pattern of aggregated proteins from *clpP* or *hslVU* mutants was similar to that of the *lon* mutant strain (data not shown).

3.3. Conclusions

Taken together, the results presented here indicate that a large fraction of the cellular proteins is unfolded during heat shock. These proteins can be refolded by chaperones [4] or degraded by proteases. Failure to be refolded or degraded results in aggregation. Our results also support previous findings [5,15–17] that the Lon (La) protease degrades the majority of the misfolded proteins, which appear in aggregates in *lon* mutants but not in wild-type cells.

In mutants lacking the ClpP-dependent proteases or the HslVU proteases the proteomes of the aggregates were similar to those of soluble proteins, similar to the pro-
teomes of aggregates in \textit{lon} mutants. However, the amount of aggregated proteins in these mutants was similar to that of the wild-type and significantly lower than that found in the \textit{lon} mutant (Fig. 1). The similarities in the pattern of protein aggregates among the mutants in proteases indicate that the proteases have low substrate specificity. Yet, the minor differences between the aggregates from the three mutants open up the possibility of identifying protease-specific substrates, which will be found in aggregates only in mutants lacking the corresponding specific protease.

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References


